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Research Article

ATTENUATION OF STREPTOZOTOCIN-INDUCED DIABETIC NEPHROPATHY IN WISTAR RATS BY ETHANOLIC BARK EXTRACT OF *ANTHOCEPHALUS CADAMBA* WITH PYRIDOXINE VIA REDUCTION OF OXIDATIVE STRESS

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ABSTRACT

Objective: A significant microvascular consequence of diabetes mellitus, diabetic nephropathy (DN) causes end-stage renal disease and progressive renal impairment. Oxidative stress and inflammation are major contributors in developing DN. In this research, ethanolic bark extract of *Anthocephalus cadamba* (200 and 400 mg/kg) with or without pyridoxine (100 mg/kg) was tested for its nephroprotective efficacy against DN in Wistar rats produced by streptozotocin (STZ).

Methods: STZ (45 mg/kg) was injected intraperitoneally once, causing diabetes. Eight groups of diabetic rats received oral treatment for 8 weeks with either pyridoxine (100 mg/kg), *A. cadamba* extract (200 or 400 mg/kg), or both. Renal function was examined by measuring serum creatinine and blood urea nitrogen (BUN). Antioxidant indices, like catalase (CAT), glutathione (GSH), and superoxide dismutase (SOD), were quantified in kidney homogenates. One-way ANOVA and Dunnett's test were used to evaluate the collected data. Hematological examination of the kidney tissue was also carried out.

Results: STZ administration significantly elevated BUN and serum creatinine levels ($p<0.001$) and reduced antioxidant enzyme activities in renal tissue compared to normal controls. Treatment with *A. cadamba* extract, especially in combination with pyridoxine, significantly ($p<0.001$) reduced serum creatinine and BUN levels while restoring renal GSH, CAT, and SOD levels toward normal. Histopathological examination revealed that the extract markedly improved renal architecture and reduced tubular degeneration and interstitial inflammation in diabetic rats. The nephroprotective effect of *A. cadamba* bark extract may be attributed to its rich phytochemical composition, particularly flavonoids and triterpenoids, which enhance antioxidant defense and attenuate oxidative stress–induced renal injury. Pyridoxine co-administration further potentiated these protective effects, likely through synergistic antioxidant mechanisms.

Conclusion: Ethanolic bark extract of *A. cadamba*, alone and in combination with pyridoxine, exhibits significant nephroprotective effects against STZ-induced DN in rats by reducing oxidative stress and improving renal function. These results suggest the therapeutic potential of *A. cadamba* as a complementary approach in managing diabetic kidney complications.

Keywords: *Anthocephalus cadamba*, Diabetic nephropathy, Oxidative stress, Pyridoxine, Antioxidant enzymes, Streptozotocin.

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INTRODUCTION

Diabetic nephropathy (DN) is characterized by elevated urine albumin excretion, reduced glomerular filtration rate (GFR), and diabetic glomerular lesions. Due to this, people with diabetes often develop severe diseases, including retinopathy, neuropathy, and nephropathy [1]. Prolonged hyperglycemia increases oxidative stress and triggers the polyol pathway, which may result in kidney damage and inflammation [2]. About one-third of diabetic people experience its development. According to reports, 25–40% of diabetic patients develop DN, which rises by 6%/year. Numerous studies have shown that renal cell injury and dysfunction may result from an increase in reactive oxygen species (ROS) generation in renal tissue [3]. Consequently, DN may be lessened by reducing this rise in ROS by increasing cellular antioxidants, like GSH, CAT, and SOD. Diabetes-related nephropathy does not seem to have a therapy that is either curative or preventative, according to clinical data. Therefore, there is an urgent need to find medications that can slow down the progression of diabetes-related problems [4]. Preventing the onset and progression of DN is a growing clinical concern. There are several synthetic medications that have been used for the purpose of treating hyperglycemic disorders or regulating excessive levels of blood glucose [5,6]. These drugs include α-glucosidase inhibitors, biguanides, and sulfonylureas. Due to their inability to normalize the lipid profile, these medications, however, have the potential to cause severe effects (hepatotoxicity and hypoglycemia) in diabetic patients [7,8]. There does not seem to be a therapy that is successful for DN, according to clinical treatments. Hypoglycaemic plant extract has been demonstrated to reduce the severity of nephropathy in experimental animals and to alleviate renal problems [9].

In addition, Traditional Chinese Medicine (TCM) has long emphasized the holistic treatment of diabetes and its other consequences, including DN, through the regulation of "Qi," improvement of blood circulation, and attenuation of "heat and dampness" syndromes [10]. Several TCM formulations, such as *Rehmanniae Radix Preparata*, *Astragalus membranaceus*, *Salvia miltiorrhiza*, and *Panax ginseng*, have demonstrated renoprotective effects by modulating inflammation, oxidative stress, and advanced glycation end-product (AGE) accumulation [11]. Experimental and clinical studies have shown that herbal decoctions like *Shenqi Dihuang Tang* and *Tangshen Formula* can attenuate glomerulosclerosis and reduce albuminuria by targeting key molecular pathways like nuclear factor kappa-B, transforming growth factor beta-1, and nuclear factor erythroid 2-related factor 2 signaling [12]. The integration of TCM principles with modern

pharmacological approaches provides a complementary avenue for developing effective, multi-targeted therapies against diabetic kidney disease [13].

According to the World Health Organization, the majority of nations utilize traditional medicine for their main medical requirements. Compared to allopathic treatment generally, the use of traditional herbal remedies is less paternalistic and more linked to patient conceptualization [14]. Herbal extracts have a number of significant pharmacological qualities that may slow the gradual deterioration of renal function in diabetics, according to a large body of research. Furthermore, traditional herbal remedies may be utilized either in place of or in addition to regular allopathic medications [15]. Some of these herbal plants help regulate and improve problems connected to diabetes, such as nephropathy, in addition to lowering blood glucose. Among the few plant-based chemical substances used to treat DN are glycosides, alkaloids, terpenoids, and flavonoids [16].

Pyridoxine is a cofactor in around 150 different processes that control the metabolism of glucose, lipids, amino acids, DNA, and neurotransmitters. Furthermore, it functions as an antioxidant by preventing the production of ROS and AGEs [17]. Antioxidants in rats induced to develop diabetes by streptozotocin (STZ) provide strong protection against damage caused by ROS. In accordance with the findings of epidemiological and experimental research, there is a clear inverse link between the levels of vitamin B6 and diabetes, and there is a definite protective effect of vitamin B6 against diabetic sequelae [18].

A plant that is utilized as an anti-diabetic is called *Anthocephalus cadamba*, which is also known as *kadamba*. This plant is a member of the family *Rubiaceae*. Triterpenes, triterpenoid glycosides, saponins, and indole alkaloids are the primary components found in the leaves of *A. cadamba*. In addition, cadambine, 3α-cadamine, dihydrocadambine, isocadamine, and isodihydrocadambine are also extracted from the leaves [19]. Therefore, to give more meaningful information, it is required to conduct an evaluation of the impact that antioxidant treatment has on DN. According to the findings of several studies, treatments that have the ability to improve antioxidant levels are useful for DN [20]. In light of this background information, the hypothesis that we tested in this research was that the combination of pyridoxine and *A. cadamba* bark extract may improve the renal dysfunction that was caused by STZ in Wistar rats diagnosed with diabetes [21].

MATERIALS AND METHODS

Plant material

The bark of *A. cadamba* (Roxb.) Miq. was collected in February 2023 from rural areas of Mathura district, Uttar Pradesh, India (27.49° N latitude and 77.67° E longitude). The plant material was taxonomically identified and authenticated at the Raw Materials Herbarium and Museum, Delhi (RHMD), CSIR–National Institute of Science Communication and Policy Research (CSIR-NIScPR), Vigyan Sanchar Bhawan, Dr. K. S. Krishnan Marg, Pusa, New Delhi-110012, India. Authentication was performed under Authentication No. NIScPR/RHMD/Consult/2023/4431-32 dated 24 April 2023, confirming the sample as the dried stem bark of *Neolamarckia cadamba* (Roxb.) Bosser (syn. *A. cadamba* Miq.; family Rubiaceae), commonly known as Kadam. A voucher specimen has been deposited in the RHMD, CSIR-NIScPR, New Delhi, under Voucher Specimen No. RHMD-AC-2023-4431-32. The new bark samples were cleaned completely with running tap water to remove any dirt or contaminants that had adhered to them, and then they were rinsed with distilled water. This operation was done after the authenticating process was completed. To avoid the destruction of heat-sensitive phytoconstituents, the bark that had been washed was allowed to air-dry in the shade at an ambient room temperature of 25±2°C for a period of 10–15 days. Following the drying process, the material was ground into a coarse powder using a mechanical grinder, then it was put through a sieve with a 40-mesh sieve ≈ 425 μm particle size, and finally it was placed in an airtight container made of amber-colored glass to preserve it from light and moisture until further extraction and phytochemical analysis.

Preparation of *A. cadamba* bark extract

The bark of *A. cadamba* (Roxb.) was shade-dried at ambient temperature and coarsely powdered using a mechanical grinder. Approximately 250 g of the powdered material was subjected to extraction with 50% ethanol using a Soxhlet apparatus for a duration of 2–3 h. The hydroalcoholic solvent was chosen to ensure the efficient extraction of both polar and non-polar phytoconstituents, including alkaloids, flavonoids, terpenoids, tannins, and saponins, which are known for their antioxidant and nephroprotective properties. After passing through Whatman No. 1 filter paper, the resulting extract was concentrated using a rotary evaporator at a regulated temperature (40–45°C) under decreased pressure to stop thermolabile chemicals from degrading. The semi-solid mass was further dried under vacuum to yield a dark brown crystalline extract. The yield percentage was calculated based on the dry weight of the extract obtained relative to the initial plant material used (34.8 g; 13.92%). The dried extract was stored in an airtight container at 4°C to protect it from moisture and light until further use in experimental assays. For *in vivo* administration, the required quantity of the dried extract was reconstituted in 1% carboxymethyl cellulose (CMC) to obtain a uniform suspension. The suspension was freshly prepared before each administration and administered orally to the experimental animals using a gastric cannula to ensure accurate dosing.

Preliminary phytochemical screening

To determine the existence of several classes of secondary metabolites that are responsible for *A. cadamba's* pharmacological actions, a qualitative phytochemical study of the ethanolic bark extract was conducted. The screening was performed using standard qualitative procedures as described by Harborne (1998) and Trease and Evans (2002), which involve specific colorimetric and precipitation tests for different phytochemical groups.

The extract was tested for the presence of saponins, alkaloids, glycosides, flavonoids, carbohydrates, terpenoids, sterols, tannins, and proteins using the following methods:

- Test for Alkaloids: Mayer's and Dragendorff's reagents were used to detect alkaloids. The formation of a creamy white (Mayer's) or orange-brown (Dragendorff's) precipitate determined alkaloid's presence.
- Test for Carbohydrates: The confirmation of the existence of carbohydrates was achieved using Molisch's test, which included the incorporation of concentrated sulfuric acid and α-naphthol, resulting in a violet ring formation when the two layers were brought together.
- Test for Sterols and Triterpenoids: The Liebermann–Burchard test was carried out by means of the addition of acetic anhydride and concentrated sulfuric acid to the extract. The emergence of a green or bluish-green hue showed the presence of sterols, while the appearance of a reddish or pink color indicated triterpenoid's presence.
- Test for Flavonoids: When magnesium turnings and strong hydrochloric acid were added to the extract, the Shinoda test was carried out. The appearance of a pink or red tint indicated the presence of flavonoids.
- Test for Amino Acids and Proteins: The Ninhydrin and Biuret test were used. The appearance of a violet color (Biuret) or purple coloration (Ninhydrin) confirmed the presence of proteins and amino acids.
- Test for Tannins: After adding a few drops of a solution containing 1% ferric chloride to the extract, the presence of tannins was determined by a coloring that was either blue-black or greenish in appearance.
- Test for Saponins: The froth test was performed by vigorously shaking the extract with water; persistent froth formation indicated saponin's presence.

The results of the qualitative phytochemical screening revealed the presence of sterols, alkaloids, carbohydrates, triterpenoids, proteins, flavonoids, tannins, and saponins, while glycosides were found to be

absent. The presence of these bioactive compounds indicates that *A. cadamba* bark may possess potent antioxidant and nephroprotective potential, which could contribute to its therapeutic efficacy against DN.

Acute toxicity study

According to the "Organization for Economic Co-operation and Development's" guideline No. 423 (Acute oral toxicity – Acute toxic class method), Swiss albino mice were used to evaluate the ethanolic bark extract of *A. cadamba* for acute oral toxicity. To conduct the research, we chose male or female mice that were healthy, and the female mice were not pregnant. Their weight ranged from 20 to 25 g. During the acclimatization process, the animals were subjected to conventional laboratory settings, which included a relative humidity of 45–55%, a temperature of 25±2°C, and a light/dark cycle of 12 h. They also have unlimited, ad libitum access to food and drink. The animals fasted for 12 h before the dosing and were allowed unlimited access to water. Oral administration of the ethanolic bark extract was performed in a series of increasing dosages of 100, 200, 500, 1000, 2000, and 4000 mg/kg body weight. 1% CMC was used to suspend the extract. Each of the groups included three different creatures. Immediately after injection, the animals were carefully monitored for any indications of toxicity or behavioral abnormalities. This monitoring continued for the first 12 h and then continued at intervals of 24 h for a total of 14 days in a row.

During the observation period, the animals were monitored for any clinical signs such as alterations in eyes, fur, skin, and mucous membranes, changes in locomotor activity, salivation, tremors, convulsions, lethargy, sleep, and coma. Mortality, if any, was recorded. Body weights were measured initially and at the end of the study to assess any significant loss or gain related to toxicity. No mortality or visible signs of toxicity were seen in the treated groups up to the maximum dose of 4000 mg/kg body weight, revealing that the ethanolic bark extract of *A. cadamba* is safe and non-toxic at the tested doses. Hence, one-tenth of the highest dose (400 mg/kg) was selected as the maximum dose for subsequent pharmacological evaluations. Following established toxicological study design guidelines, 400 mg/kg (one-tenth of the maximum tested dose) was used to ensure a suitable safety margin while being sufficiently elevated to identify potential treatment-related adverse effects in the primary investigation.

Induction of diabetes mellitus

Before the induction of diabetes, all experimental animals were given unlimited access to water to avoid dehydration and were fasted for 12 h. One intraperitoneal injection of STZ at a dosage of 45 mg/kg body weight, freshly dissolved in 0.1 M cold sodium citrate buffer (pH 7.4), developed diabetes mellitus [22]. The solution was prepared immediately before administration to ensure the stability of STZ. Following injection, animals were provided with 5% glucose solution for 24 h to prevent hypoglycemia due to the transient hyperinsulinemia that follows β-cell destruction. Seventy-two hours after induction, a glucometer was used to measure the fasting blood glucose levels after blood samples were drawn from the tail vein [23]. Only rats with fasting blood glucose levels of 250 mg/dL or above were classified as diabetics and included in the study. The diabetic state was maintained and confirmed throughout the study by periodic blood glucose monitoring. Control animals received only the vehicle (sodium citrate buffer, pH 7.4) and were maintained under identical conditions [24].

Experimental design

Following the successful induction of diabetes mellitus in the animals, the animals were divided into eight groups with 8 rats at random. The rats were precisely weighed and distributed to guarantee that the body weight Table 1 was consistent throughout. Every single therapy was given orally once every day for a period of 8 weeks in a row utilizing an oral gavage.

To guarantee equal dispersion and stability, each dosage was newly prepared in a suspension of 1% carboxymethylcellulose (CMC) before being administered. During the treatment period, all rats were kept in the laboratory under normal settings, which included a relative humidity of 50–60%, a temperature of 25±2°C, and a light/dark cycle of 12 h. In addition, they were given a standard pellet meal, and water was available to them whenever they desired.

An examination of the nephroprotective effectiveness of ethanolic bark extract of *A. cadamba* against STZ-induced DN in Wistar rats was the purpose of the experimental design. This evaluation was conducted both on its own and in conjunction with pyridoxine.

Assessment of STZ-induced DN

Estimation of blood urea nitrogen (BUN) and serum creatinine

BUN and serum creatinine levels were estimated using commercially available diagnostic kits. Serum creatinine was measured using the alkaline picrate (Jaffe's) method with a Creatinine Kit (Catalog No. CR-510; Erba Diagnostics Mannheim GmbH, Germany), following the manufacturer's protocol, with absorbance measured at 520 nm. BUN was determined using an enzymatic Urea/BUN Kit based on the urease–glutamate dehydrogenase (GLDH) method (Catalog No. UR-220; Transasia Bio-Medicals Ltd., Mumbai, India), with absorbance recorded at 540 nm. All assays were performed in triplicate using freshly separated serum samples, and concentrations were expressed as mg/dL.

Using commercial diagnostic kits, the levels of serum creatinine and BUN were measured. Standard enzymatic and colorimetric techniques served as the foundation for the assays:

- Creatinine concentration was determined using the alkaline picrate method (Jaffe's reaction), in which creatinine forms an orange-red chromogen with picric acid under alkaline conditions, and absorbance was determined spectrophotometrically at 520 nm.
- Using the urease–glutamate dehydrogenase technique, which produces ammonia and carbon dioxide when urease hydrolyzes urea, BUN was calculated. After that, the quantity of ammonia generated was measured at 540 nm using spectrophotometry.

The data were represented as mg/dL of serum, and all measurements were carried out in triplicate to guarantee accuracy. To determine whether there were any notable variations between the experimental groups, the data were statistically examined.

Preparation of renal homogenate

Following sacrifice, both kidneys were carefully removed, thoroughly cleaned with ice-cold isotonic saline to get rid of any blood or debris that could have adhered, and then blotted dry with

Table 1: Experimental grouping and treatment schedule of Wistar rats

Groups	Treatment description	Treatment details	Dose (mg/kg, p.o.)	No. of rats
I	Normal Control	Rats without diabetes were given simply the vehicle (1% CMC).	—	8
II	Diabetic Control	STZ-induced diabetic rats received no treatment	—	8
III	Metformin Positive Control	Diabetic rats administered metformin	100	8
IV	Low-Dose Extract	Diabetic rats administered *A. cadamba* bark extract	200	8
V	High-Dose Extract	Diabetic rats administered *A. cadamba* bark extract	400	8
VI	Pyridoxine	Diabetic rats administered pyridoxine	100	8
VII	Low-Dose Combination	Diabetic rats administered *A. cadamba* extract+pyridoxine	200+100	8
VIII	High-Dose Combination	Diabetic rats administered *A. cadamba* extract+pyridoxine	400+100	8

The duration of treatment (8 weeks), CMC: 1% carboxymethyl cellulose (CMC), P.O: Per Oral

filter paper. Every kidney was precisely weighed and split in half, where the first half was fixed in 10% neutral buffered formalin, while the other half was used for biochemical calculations. To avoid enzymatic degradation, the kidney piece that was selected for biochemical analysis was finely chopped using sterile scissors and homogenized in 10% (w/v) ice-cold phosphate buffer (0.1 M, pH 7.4) using a Teflon-glass homogenizer in a cold environment. To generate a clear supernatant, the resultant homogenate was subjected to centrifugation at 10,000 rpm at 4°C for 15 min. Before further measurement of antioxidant enzyme characteristics, such as CAT, GSH, and SOD, the supernatant was meticulously collected and kept at -20°C.

Estimation of protein content

Total protein concentration in renal tissue homogenates was determined using the Bradford protein assay with Bovine Serum Albumin (BSA; Catalog No. A2153, Sigma-Aldrich, USA) as the standard. Absorbance was measured at 595 nm using a UV–visible spectrophotometer. Antioxidant enzyme activities, including catalase (CAT) and superoxide dismutase (SOD), were normalized to protein content and expressed as units per milligram of protein (U/mg protein).

Determination of oxidative stress markers

Estimation of reduced glutathione (GSH)

The method used to measure the quantity of GSH in renal tissue was based on the reaction of GSH with '5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)', that resulted in a yellow-colored chromophore visible at 412 nm. One milliliter of kidney tissue homogenate (100 mg/mL in phosphate buffer, pH 7.4), 0.2 mL of 50% trichloroacetic acid (TCA), and 0.8 mL of distilled water were mixed together in a 15- mL test tube. After 10–15 min of periodic agitation to allow the protein to precipitate, the mixture was subjected to centrifugation at 3000 rpm for 15 min to separate out the clear supernatant. After that, 0.6 mL of the supernatant was passed to a clean tube, after which was mixed with 20 µL of 0.1 M DTNB solution and 0.8 mL of 0.4 M Tris buffer with a pH of 8.9 that had been made in 100% methanol. To facilitate the formation of color, the mixture was given a little shake after the addition of DTNB, and then it was left to incubate at 25°C for 5 min. The absorbance of the yellow-colored solution that was produced was measured using spectrophotometry at a wavelength of 412 nm. This measurement was done in comparison to a reagent blank that included all of the reagents except for tissue homogenate. The researchers determined the concentration of GSH in the kidney samples using a standard curve created from known quantities of standard reduced GSH, expressing it as nanomoles (nmol) of GSH per gram of wet tissue weight. The procedure allowed for the determination of the concentration of GSH in the kidney samples.

Estimation of CAT

The dichromate-acetic acid reagent technique was used to accomplish the task of determining the level of CAT activity present in the kidney tissue. With the kidney tissue homogenate, 1.0 mL of phosphate buffer (0.1 mM) and a pH of 7.4 was combined. The introduction of a solution of hydrogen peroxide (H_2O_2) was the catalyst that set off the enzymatic process. A total of 2.0 mL of dichromate-acetic acid reagent was mixed with the reaction mixture to bring the reaction to a halt at predetermined time intervals of 0, 30, and 60 s [25].

To produce a blank medium, 1.6 mL of phosphate buffer and 2.0 mL of dichromate-acetic acid reagent were combined in a separate tube. The addition of tissue homogenate was not included in this process. After that, the test tubes and the blank tubes were heated for 10 min in a water bath to form a complex that was green in color. The green hue was produced due to the subsequent reduction of dichromate by the H_2O_2 that was still present. After the tubes had been heated, they were rested at room temperature (25°C), and then an ultraviolet–visible spectrophotometer was employed to determine the absorbance at 570 nanometers [26]. This procedure was done after the tubes had been heated. For every milligram (mg) of protein, the CAT activity was expressed in micromoles (µmol) of hydrogen peroxide broken down per minute. This measurement serves as an indication of the enzyme's ability to neutralize hydrogen peroxide, which in turn reflects the antioxidative defense state of the renal tissue. CAT activity was expressed as µmol of H_2O_2 decomposed per minute per milligram of protein (U/mg protein).

Assessment of SOD activities

The efficacy of SOD in kidney tissue homogenate to prevent superoxide radicals produced in the reaction mixture from reducing nitro blue tetrazolium (NBT) to blue-colored formazan was assessed. This was done to determine the level of SOD activity in kidney tissue homogenate [27]. For the purpose of determining the absorbance of the chromogen that was created, a spectrophotometric measurement was carried out at a wavelength of 560 min. SOD activity was expressed as units per milligram of protein (U/mg protein), where one unit represents 50% inhibition of NBT reduction.

Procedure

The reaction mixture consisted of about 0.1 mL of NADH (780 µM), 0.15 mL of NBT (300 µM), 0.2 mL of tissue homogenate, 0.05 mL of phenazine methosulfate (PMS, 186 µM), 1.5 mL of sodium pyrophosphate buffer (0.052 mM, pH 8.3), and 0.4 mL of distilled water. The total volume of the reaction mixture was approximately 1.5 mL. With the addition of PMS, the reaction was kicked off, and it was then incubated at 30°C for 60 s [28]. Immediately after the incubation period, the reaction was halted by adding 0.5 mL of glacial acetic acid. The mixture was then violently agitated with 2.0 mL of n-butanol to extract the chromogen. The mixture was centrifuged at 3000 revolutions/min for 10 min after it had been allowed to rest for 10 min to separate the butanol layer [29]. At a wavelength of 560 nm, a spectrophotometer was used to determine the color intensity of the chromogen that was present in the butanol layer. The reference blank was n-butanol, and the control was an enzyme-free system. Under the circumstances of the experiment, the SOD activity was expressed as the quantity of enzyme that was important to achieve a 50% inhibition of the reduction of NBT. Small adjustments have been made to the idea that Khalaj L *et al*. (1984) outlined, which serves as the foundation for this strategy [30].

Histopathological examination

All the animals were killed in a humane manner by having their cervical vertebrae dislocated at the conclusion of the experiment. Immediately after the kidneys were removed, they were rinsed gently with normal saline to eliminate any blood residues, and then they were preserved in 10% neutral buffered formalin for histological analysis [31]. The preserved tissues were then washed with xylene, dehydrated using progressively higher ethanol concentrations, and ultimately embedded in paraffin wax. To achieve fine slices with a thickness of roughly 4–5 µm, a rotary microtome was used, and these sections were then put on clean glass slides. To facilitate the study of the slices under a microscope, hematoxylin and eosin (H&E) were considered for the staining [32].

To evaluate histological changes, the stained sections were inspected under a light microscope at magnifications of 10× and 40× to identify the glomerular damage, tubular necrosis, cellular infiltration, and interstitial inflammation. The microscope was equipped with a digital camera to capture photomicrographs. Scale bars represent 20 µm for reference calibration [33].

To enable objective and reproducible assessment of renal injury, histopathological changes were additionally evaluated using a semi-quantitative scoring system. Renal sections were scored independently by a blinded observer for key pathological parameters, including tubular injury, glomerular damage, and interstitial inflammation, using a four-point scale as follows: 0=Normal histology; 1=Mild changes (<25% area affected); 2=Moderate

changes (25–50% area affected); and 3=Severe changes (>50% area affected). Tubular injury was assessed based on epithelial degeneration, tubular dilation, and necrosis; glomerular damage was evaluated by mesangial expansion and glomerular distortion; and interstitial inflammation was graded according to inflammatory cell infiltration and interstitial edema. Mean histological scores were calculated for each group and used for comparative analysis alongside qualitative observations.

In addition to qualitative histological assessment, renal injury was evaluated using a semi-quantitative scoring system. Glomerular damage, tubular degeneration/necrosis, interstitial inflammation, and cellular edema were graded on a scale of 0–4 as described. Scores were assigned based on the percentage of tissue involvement. At least 10 randomly selected, non-overlapping microscopic fields per section were analyzed in a blinded manner, and mean injury scores were calculated for each animal.

Statistical analysis

All experimental data are expressed as mean±standard error of the mean (SEM). Prior to statistical analysis, the data were assessed for normality and homogeneity of variance using appropriate tests. Since the data satisfied the assumptions required for parametric analysis, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post hoc test was applied to compare treatment groups with the diabetic control group. GraphPad Prism (version 5.0, GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. A $p<0.05$ was considered statistically significant. $p<0.05$ was used as the study's statistical significance level. For graphical reasons, statistical significance was indicated by the following values: $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).

This approach ensured the reliability and reproducibility of results by comparing multiple treatment groups with the diabetic control, while maintaining control over Type I error through Dunnett's multiple comparison analysis.

RESULTS

The phytochemical constituents of the ethanolic bark extract of *Anthocephalus cadamba* was showed in Table 2.

Effect of ethanolic bark extract of A. cadamba on serum creatinine in STZ-induced DN rats

The experimental rats showed significantly higher level of creatinine (3.045±0.295) in the blood serum as compared to the control group(0.960±0.059). The STZ-induced diabetic rats that were given pyridoxine at a dose of 100 mg/kg showed a reduction in the amount of creatinine that was present in their blood serum. Following treatment with ethanolic bark extract of *A. cadama* at 200 mg/kg and 400 mg/kg doses, a discernible decrease in creatinine level was seen in the blood serum. Diabetic rats that were given extract together with pyridoxine at a dose of 100 mg/kg, exhibited a significantly decreased creatinine level in their blood serum, as shown in Fig. 1.

Effect of ethanolic bark extract of *A. cadamba* on BUN in STZ-induced DN rats

In comparison to the control group (29.80±1.497), the experimental rats exhibited a considerable rise in the amount of BUN (58.00±2.966) that was present in their serum. The STZ-diabetic rats that were given pyridoxine at a dose of 100 mg/kg demonstrated a reduction in the amount of BUN in their blood serum. Following administration of the ethanolic bark extract of *A. cadama*, statistically significant reductions in BUN levels were seen in the serum of the subjects. Rats with STZ diabetes that were given ethanolic bark extract of *A. cadamba* at doses of 200 mg/kg and 400 mg/kg, together with pyridoxine at a dose of 100 mg/kg, had a more substantial reduction in the amount of urea nitrogen (BUN) in their blood serum, as shown in Fig. 2.

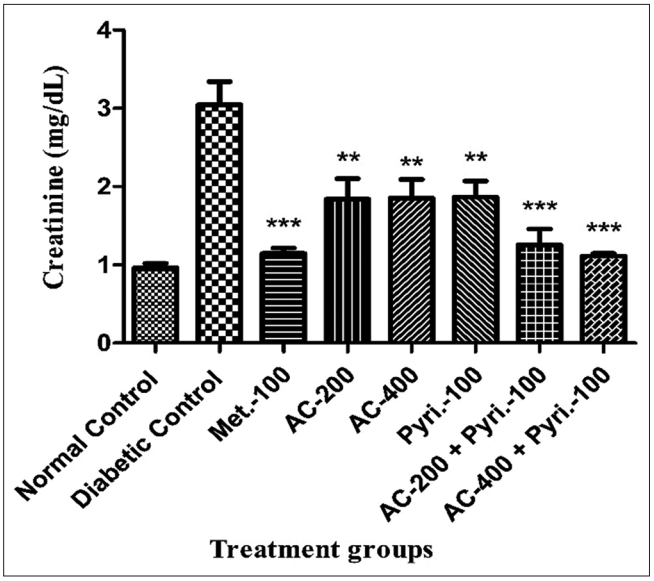

Fig. 1: Effect of ethanolic bark extract of *Anthocephalus cadamba* and pyridoxine on serum creatinine levels (mg/dL) in streptozotocin (STZ)-induced diabetic control group rats. Data are expressed as mean±standard error of the mean (n=8). Treatment groups were compared with the diabetic control group using one-way ANOVA followed by Dunnett's multiple comparison test. p<0.05 (), p<0.01 (), and p<0.001 ()

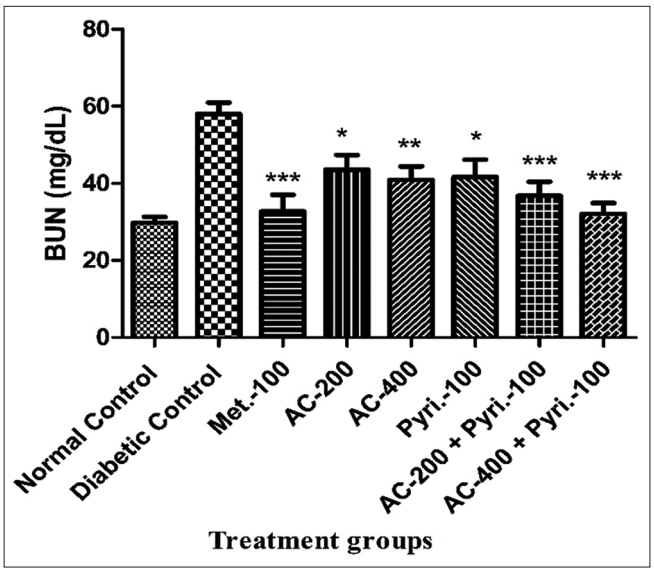

Fig. 2: Effect of ethanolic bark extract of *Anthocephalus cadamba* and pyridoxine on blood urea nitrogen (BUN) levels (mg/dL) in streptozotocin (STZ)-induced diabetic control group rats. Data are expressed as mean±standard error of the mean (n=8). Treatment groups were compared with the diabetic control group using one-way ANOVA followed by Dunnett's multiple comparison test. p< 0.05 (), p<0.01 (), and p<0.001 (). BUN: Blood urea nitrogen

Effect of ethanolic bark extract of *A. cadamba* on renal oxidative stress markers in STZ-induced DN rats

Effect on reduced GSH levels

The diabetic control group showed lower GSH (16.40±3.121) level in comparison to the normal control group(48.00 ±4.967). The GSH level was found to be substantially elevated ($p<0.05$) in the groups that had been treated with ethanolic bark extract for a period of 8 weeks.

A significantly higher increase ($p<0.01$) in the level of GSH was seen. Diabetic rats treated with ethanolic bark extract of *A. cadamba* (200 mg/kg and 400 mg/kg) with pyridoxine (100 mg/kg) showed a more significant increase in GSH level in renal Fig. 3.

Effect on CAT levels

The effect of the ethanolic bark extract of *A. cadamba* on renal catalase (CAT) levels was evaluated in STZ-induced diabetic rats. The diabetic control group showed lower CAT level (0.375±0.070)in comparison to the normal control group(2.445±0.468). In comparison to the diabetic control group, the CAT level was found to be substantially elevated ($p<0.05$) in the ethanolic bark extract-treated groups for a period of 8 weeks. A significantly higher increase ($p<0.01$) in the level of CAT was seen. STZ-diabetic rats treated with ethanolic bark extract of *A. cadamba* (200 mg/kg and 400 mg/kg) with pyridoxine (100 mg/kg) showed a more significant increase in CAT level in renal Fig. 4.

Effect on SOD levels

The effect of the ethanolic bark extract of *A. cadamba* on renal catalase (CAT) levels was evaluated in STZ-induced diabetic rats. The diabetic control group showed a lower SOD (5.167±1.249) level in comparison to the normal control group(18.00 ±3.235). The SOD level was found to be substantially elevated ($p<0.05$) in the treatment groups for a period of 8 weeks when compared to the diabetic control group. A significantly higher increase ($p<0.01$) in the level of SOD was seen. STZ-diabetic rats treated with ethanolic bark extract with pyridoxine (100mg/kg) showed more significant increase in SOD level in renal Fig. 5.

Effect of ethanolic bark extract of *A. cadamba* on renal histopathological changes

To determine whether or not *A. cadamba* has a protective function against physiological impairment, H&E staining was carried out. During the histological testing, the kidney tissue from the control group displayed a normal cell architecture. In contrast, the kidneys of diabetic rats exhibited significant tubular degeneration or necrosis, extreme degrees of proximal tubule, cellular edema in renal tubules, and interstitial inflammation. Severe cases demonstrated these findings. In spite of this, the severity of renal damage was reduced by ethanolic bark extract of *A. cadamba*, as well as by combining it with pyridoxine (100 mg/kg) and metformin (100 mg/kg) (Fig. 6).

Semi-quantitative histopathological injury scoring revealed a significant increase in renal damage in STZ-induced diabetic rats compared to the normal control group ($p<0.001$). Treatment with ethanolic bark extract of *A. cadamba* produced a dose-dependent reduction in renal

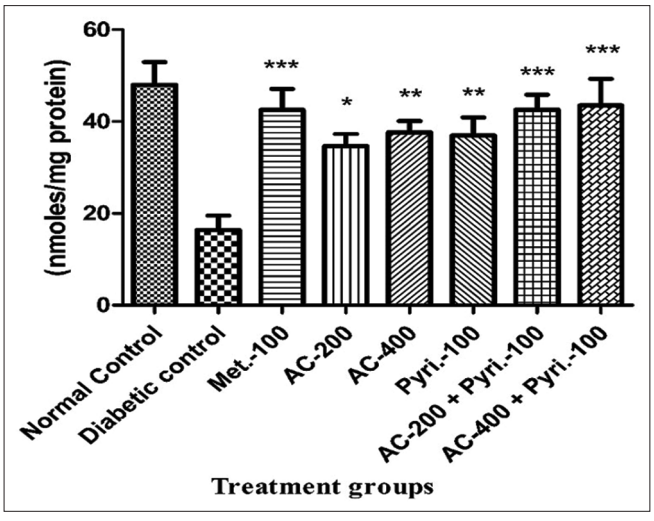

Fig. 3: Effect of ethanolic bark extract of *Anthocephalus cadamba* and pyridoxine on reduced glutathione (GSH) levels (nmol/g tissue) in kidney tissue homogenates of streptozotocin (STZ)-induced diabetic control group rats. Data are expressed as mean±standard error of the mean (n=8). Treatment groups were compared with the diabetic control group using one-way ANOVA followed by Dunnett's multiple comparison test. p< 0.05 (*), p<0.01 (**), and p<0.001 (***). GSH: Reduced glutathione

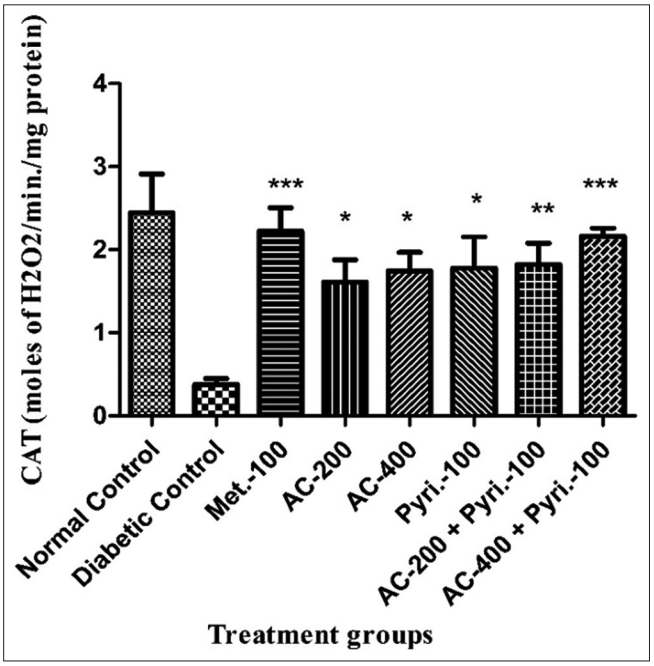

Fig. 4: Effect of ethanolic bark extract of *Anthocephalus cadamba* and pyridoxine on CAT activity (U/mg protein) in kidney tissue homogenates of streptozotocin (STZ)-induced diabetic control group rats. Data are expressed as mean±SEM (n=8). Treatment groups were compared with the diabetic control group using one-way ANOVA followed by Dunnett's multiple comparison test. p< 0.05 (*), p<0.01 (**), and p<0.001 (***). CAT: Catalase

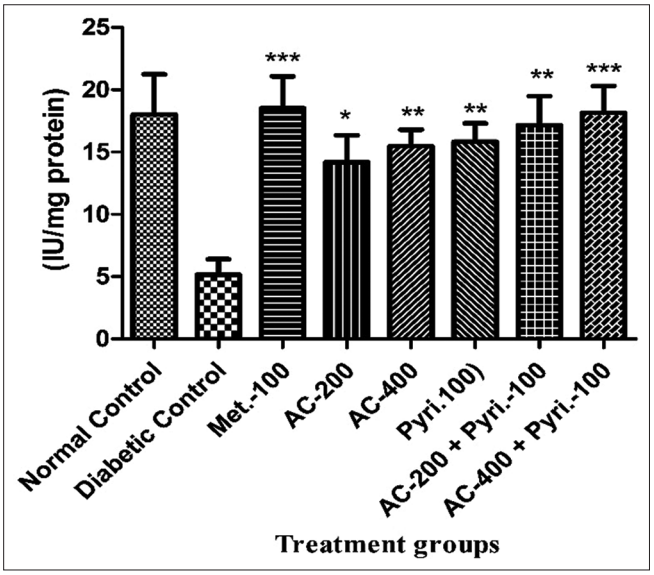

Fig. 5: Effect of ethanolic bark extract of *Anthocephalus cadamba* and pyridoxine on SOD activity (U/mg protein) in kidney tissue homogenates of streptozotocin (STZ)-induced diabetic control group rats. Data are expressed as mean±SEM (n=8). Treatment groups were compared with the diabetic control group using one-way ANOVA followed by Dunnett's multiple comparison test. p< 0.05 (*), p<0.01 (**), and p<0.001 (***). SOD: Superoxide dismutase

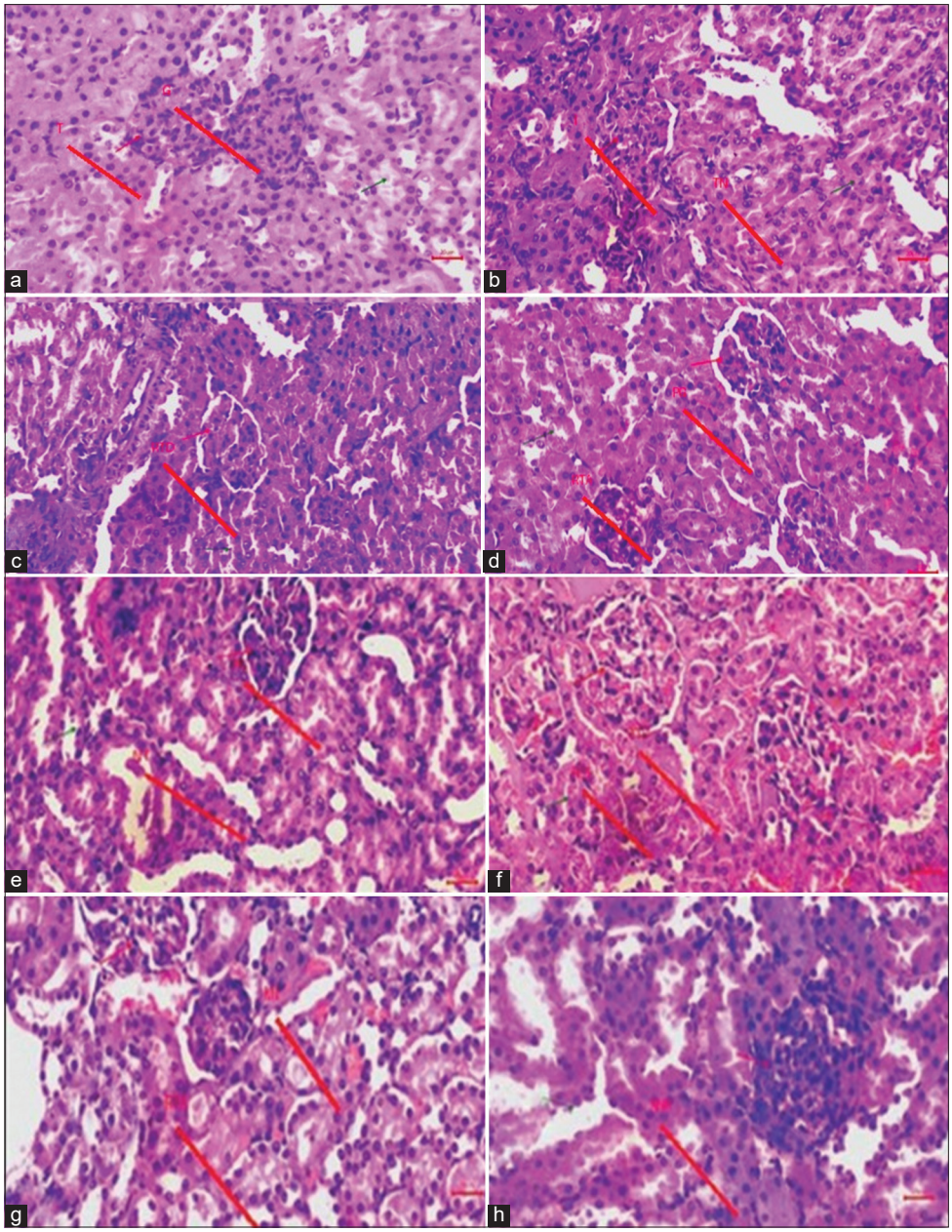

Fig. 6: Representative photomicrographs of rat kidney sections stained with hematoxylin and eosin (H&E) and examined under a light microscope at 400× magnification. Scale bar=20 μm (shown on each panel). (a) Normal control group showing intact glomeruli and normal renal tubules. (b) Diabetic control group showing tubular necrosis, glomerular damage, cellular edema, and marked interstitial inflammatory infiltration. (c) Metformin-treated group (100 mg/kg) showing partial restoration of renal architecture. (d) *Anthocephalus cadamba* bark extract–treated group (200 mg/kg) showing moderate improvement in tubular structure. (e) *A. cadamba* bark extract–treated group (400 mg/kg) showing near-normal renal architecture. (f) Pyridoxine-treated group (100 mg/kg) showing attenuation of tubular damage. (g) Combination-treated group (A. cadamba 200 mg/kg + pyridoxine 100 mg/kg) showing marked renal recovery. (h) Combination-treated group (A. cadamba 400 mg/kg + pyridoxine 100 mg/kg) showing almost complete normalization of renal histology. Arrows indicate key histopathological features (TN, TD, GD, I, CE, TR). Photomicrographs are representative of findings from n=8 rats per group. Data are expressed as mean±SEM. Statistical analysis was performed using One-way ANOVA followed by Dunnett's test. $p<0.05$, $p<0.01$, $p<0.001$ compared with diabetic control

injury scores. The combination of *A. cadamba* with pyridoxine showed the most pronounced protective effect, confirming and quantitatively supporting the qualitative observations seen in the representative H&E photomicrographs (Fig. 6a and b).

Arrows indicate key histopathological alterations, including tubular necrosis (TN), interstitial inflammatory infiltration (I), glomerular damage (GD), and restoration of renal architecture (RTA).

DISCUSSION

Natural medicinal plants that contain numerous phytochemicals, like glycosides, steroids, flavonoids, alkaloids, tannins, and polysaccharides, were utilized to treat a variety of illnesses from the beginning of time. In this work, we provide evidence that the ethanolic bark extract of *A. cadamba* protects against the development of DN. Due to a reduction in insulin production, STZ-induced diabetes mellitus damages the β-cells of the islets of Langerhans, causing diabetes and its associated

Table 2: Phytochemical constituents of the ethanolic bark extract of *Anthocephalus cadamba*

Phyto-constituents	Ethanol extract
Carbohydrates	+
Glycosides	-
Sterols	+
Proteins and Amino acids	+
Alkaloids	+
Tannins	+
Triterpenoids	+
Saponins	+
Flavonoids	+

Phytochemical tests of A. cadamba. Where, (+): positive (-): negative

consequences, including DN. While the ethanolic bark extract of *A. cadamba* therapy decreased blood glucose levels, plasma blood glucose levels increased during diabetes, suggesting that ethanolic bark extract of *A. cadamba* may have an impact on diabetes.

Increased oxidative stress and osmotic factors, which were identified as promoters of diabetic microvascular diseases, including DN, have been linked to anomalies like increased polyol pathway activity caused by hyperglycemia. The pathogenesis of various diabetes problems, including DN, is linked to hyperglycemia's increased generation of ROS. ROS weaken the cell's antioxidant defenses, increasing its vulnerability to oxidative damage. It also targets proteins, lipids, and DNA, causing them to oxidize, which alters the structure and function of cells. As renal hemodynamics is changed, kidney abnormalities worsen and result in proteinuria, renal failure, and glomerulosclerosis. In DN, decreased protein synthesis and a nitrogen imbalance initiate the development of nitrogenous non-protein molecules such as BUN and creatinine. Elevated blood creatinine and BUN levels in the diabetic rats indicated increasing renal damage. Creatinine and BUN levels in the serum were considerably lowered by the ethanolic bark extract of *A. cadamba*. By lowering blood creatinine and BUN levels, *A. cadamba's* ethanolic bark extract and pyridoxine provide protection against the onset of DN. By reducing the production of ROS, the ethanolic bark extract of *A. cadamba* with pyridoxine offers synergistic protection against the onset of DN.

ROS were produced in greater quantities under oxidative stress, which, on interaction with the polyunsaturated fatty acids, leads to the initiation of lipid peroxidation (LPO) in the renal tissue, causing toxicity or damage to the tissue. Superoxide radicals are reduced, oxidized, and converted to molecular oxygen and H_2O_2. Other antioxidant enzymes, including CAT, protect the tissue against the very reactive hydroxyl radical (OH) and are implicated in the secretion of H_2O_2. According to studies, peroxisomal dysfunction caused by a decrease in GSH, SOD, and CAT levels exacerbated kidney damage during DN.

Antioxidants (such as pyridoxine and vitamins C and E) prevent DN from developing. The plant possesses antioxidant properties due to the abundance of phenols and flavonoids in its bark. Excessive formation of free radicals is often a sign of diabetes mellitus, and the development of diabetic complications may be significantly accelerated by hyperglycemia-induced mitochondrial ROSs production. The plant's antioxidant properties have slowed the development of nephropathy brought on by diabetes. Histopathological analysis of kidney sections from STZ diabetic rats revealed interstitial inflammation, cellular edema, severe proximal tubule degeneration or necrosis, and renal tubules. The aforementioned changes were considerably reduced by treatment with *A. cadamba* ethanolic bark extract (200 and 400 mg/kg) and in conjunction with pyridoxine (100 mg/kg), indicating a preventive function against renal injury.

CONCLUSION

The current research revealed that ethanolic bark extract of *A. cadamba* exerts a significant nephroprotective effect in STZ-induced diabetic rats. The extract effectively restored renal antioxidant status and ameliorated biochemical and histopathological alterations associated with DN. Co-administration with pyridoxine produced an additive effect, indicating a synergistic role in mitigating oxidative renal damage. These results suggest that *A. cadamba* bark extract, particularly in combination with pyridoxine, can be a potential natural therapeutic agent for preventing or delaying the progression of DN. More research is necessary to determine the isolation and testing of the active phytoconstituents, conducting *in vitro* mechanistic studies, and performing longer-term toxicity and efficacy studies.

FUNDING

Not applicable.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest

AUTHORS' CONTRIBUTION

Talever Singh conceptualized and designed the study, conducted the experimental work, performed data acquisition and analysis, and drafted the manuscript. Saravanan K provided supervision throughout the research process, contributed to the study design and interpretation of data, and critically revised the manuscript for important intellectual content. Both authors read and approved the final version of the manuscript.

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